synt:em

Technical Report - US patent application n°09/485,571

I – Polypeptides as active substances.

1) Cytochrome C .

a) Conjugation of Cytochrome C with SynB4

Cytochrome C (CyC, 12 kDa) was incubated in phosphate buffer with 10 equivalents of Succinimidyl-Maleimido-Propionate (SMP). The resulting activated SMP-CyC was purified by Gel-Filtration. SMP-CyC was incubated with the fluorescently labeled 3-Mercapto-propionyl-K(FITC)-SynB4 (AWSFRVSYRGISYRRSR) in the same buffer, and the resulting Thio-ether conjugate CyC-SMP-3MP-K(FITC)-SynB4 was purified by Gel-Filtration. For control purposes, unconjugated CyC was labeled by incubation with Fluorescein-Isothioscyanate FITC and purified by Gel-filtration.

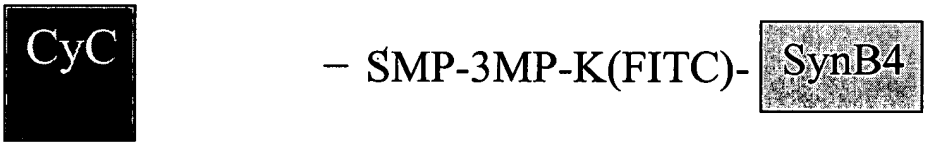

Figure 1: Schematic representation of vectorised CyC with SynB4

b) Cell uptake experiments

K562 cells (erythroleukemia cell line) were incubated with the conjugate CyC-SMP-3MP-K(FITC)-SynB4 or with FITC-CyC as a control. Cell-associated fluorescence was then assessed by flow cytometry.

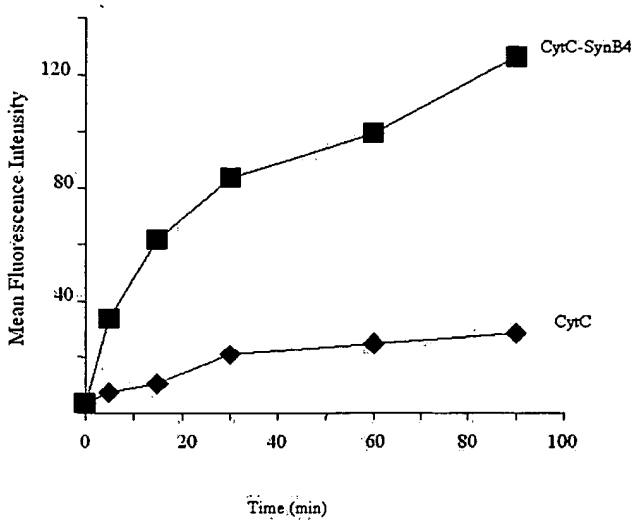

Figure 2: Cell uptake of free and vectorised cytochrome C as measured by flow cytometry.

The cell uptake of free cytochrome C was very low during the 90 min incubation. However, its coupling with SynB4 increases significantly its transport across the cell membrane. This increase, at 90 min, was about 6-fold.

2) mtb 8.4 Protein.

a) Formation of the Protein-SynB1 Complex

The mtb8.4 protein (8.4 Kd) was derivatized by adding 10 volumes of DMF, followed by addition of 10 molar equivalents of 2-Iminothiolane hydrochloride in water/DMF (1/1). About 20 equivalents of DIEA were then added. The derivatized protein was precipitated by addition of 20 volumes of diethylether, resuspended in water containing 0.1 % TFA, and submitted to gel-filtration on a Pharmacia Sephadex G10 using Water/0.1% TFA as elution solvent. The protein fractions were lyophilised. The 2IT- mtb8.4 lyophilisate was resuspended in water containing 0.1% TFA, and 10 volumes of DMF were added. About 2-5 equivalent of SPDP-peptide SynB4 (AWSFRVSYRGISYRRSR) in DMF were added, followed by 10 equivalents of DIEA. The conjugated protein was precipitated with 20 volumes of Diethylether, then submitted to gel-filtration as indicated above.

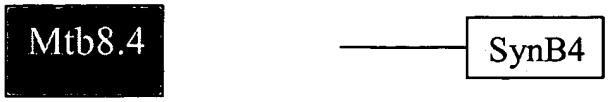

Figure 3: Schematic representation of vectorised mtb8.4 with SynB4

b) Cell uptake experiments

K562 cells were incubated with either the fluorescent free mtb8.4 or the conjugate mtb8.4-SynB4. Cell-associated fluorescence was then assessed by flow cytometry.

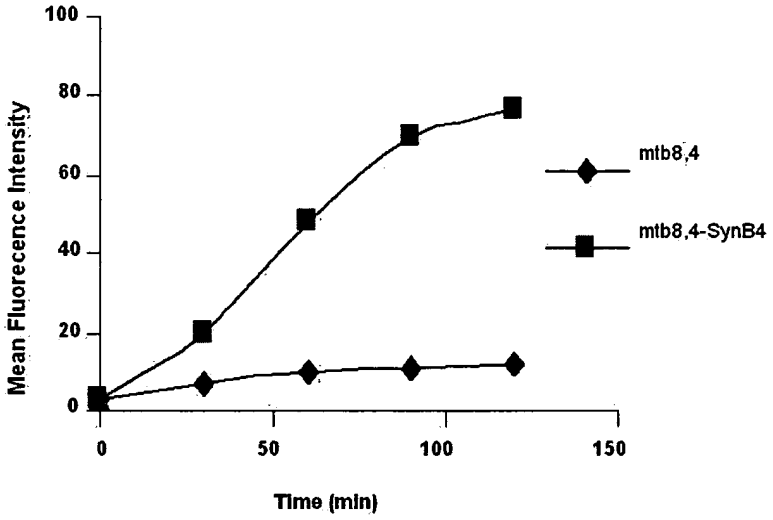

Figure 4: Cell uptake of free and vectorised mtb8.4 as measured by flow cytometry.

Vectorisation of mtb8.4 leads to a significant increase in its cell uptake. This increase, at 120 min incubation, was about 8-fold.

3) Streptavidin-Biotin.

a) Formation of the Streptavidin/Biotin-SynB1 Complex

Texas Red labeled Streptavidin (STRV; 70 kDa) was incubated for 4 hours in phosphate-buffered saline with 4 equivalents of the N-terminal biotinylated peptide vector SynB1 (RGGRLSYSRRRFSTSTGR).

Figure 5: Schematic representation of vectorised Streptavidin with SynB1

b) Cell uptake experiments

The resulting vector/streptavidin complex was incubated for 15 min with K562 cells. As a negative control, cells were incubated with the free Texas-Red Streptavidin. After incubation, Cells were washed by centrifugation and the uptake was measured by flow cytometry.

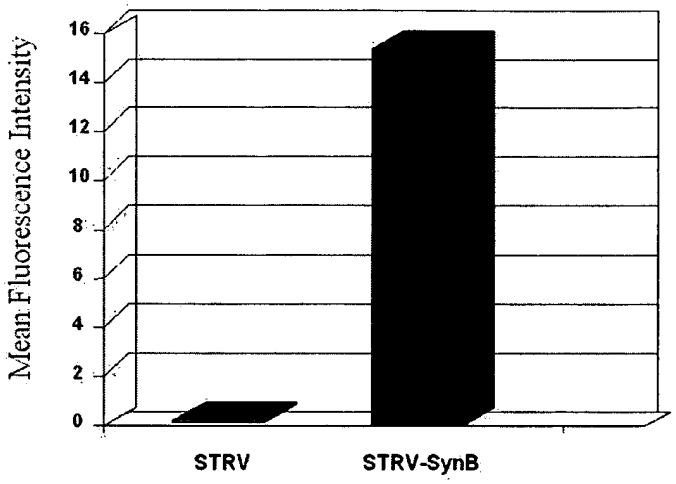

Figure 6: Cell uptake of free and vectorised streptavidin as measured by flow cytometry

Large proteins are known not to penetrate cells in an efficient manner. In fact, the data presented in this figure shows that free streptavidin (70 Kda) has a very low uptake in K562 cells. Interestingly, its vectorisation with the peptide vector SynB1 increases significantly its uptake.

II – Antibodies as active substances.

Anti-Biotin monoclonal-antibody.

a) Formation of the Vector/Antibody conjugate

N-Terminal Biotinylated SynB1 (RGGRLSYSRRRFSTSTGR) was incubated in phosphate-buffered saline for 4 hours with FITC-labeled anti-Biotin monoclonal-antibody (FITC-IgG, 150 kDa).

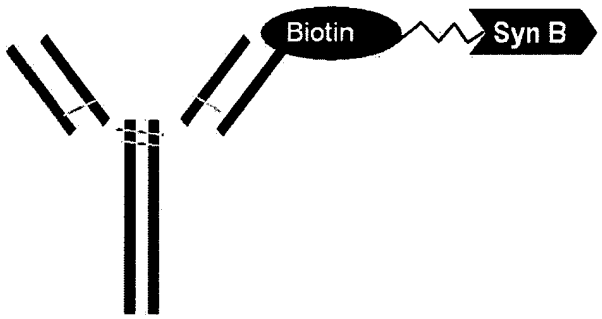

Figure 7: Schematic representation of vectorised IgG with SynB1

b) Cell Uptake experiments

The resulting vector/IgG complex was incubated for 15 min with K562 cells. As a negative control, cells were incubated with the free FITC-IgG. After incubation, cells were washed and the distribution of FITC-IgG fluorescence was measured in living cells by Flow Cytometry.

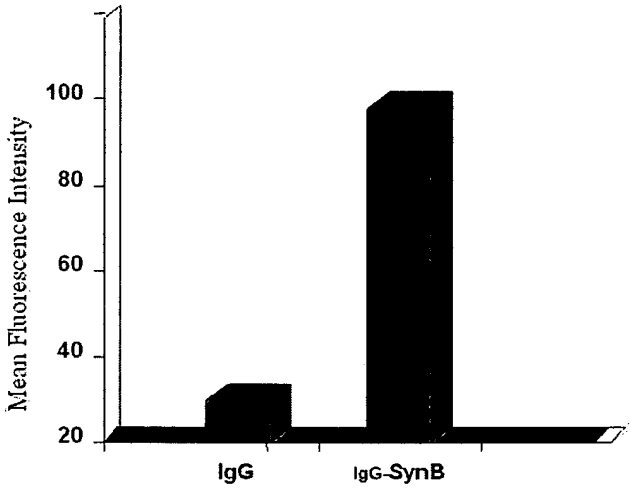

Figure 8: Cell uptake of free and vectorised IgG as measured by flow cytometry

Antibodies are known not to penetrate cells in an efficient manner. In fact, the data presented in this figure shows that IgG (150 Kda) has a very low uptake in K562 cells. Interestingly, its vectorisation with the peptide vector SynB1 increases significantly its uptake.

III – Nucleic acids as active substances.

Acycloguanosine (antivirals).

a) Coupling of Acycloguanosine to SynB3 via a succinylester link

Acycloguanosine (ACY) was first derivatized in Dimethysufoxide (DMSO) by succinic anhydride in the presence of Dimethylaminopyridine (DMAP) and Diisopropylethlamine (DIEA). This intermediate ACY-Hemisuccinate (ACY-OSUC) was purified by Reverse-Phase High Performance Liquid Chromatography. The peptide vector SynB3 was coupled to ACY-OSUC using PyBOP as an activator in the presence of DIEA. The resulting conjugate ACY-OSUC-SynB3 was purified by Reverse-phase High Performance Liquid Chromatography.

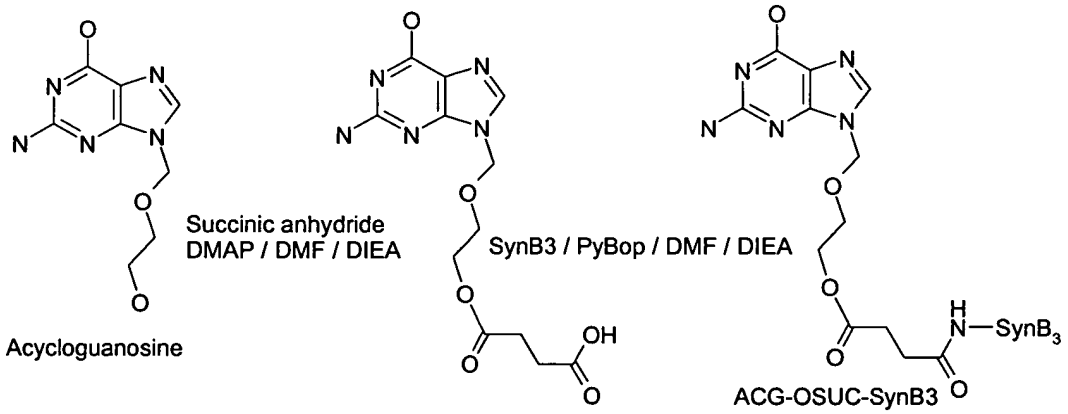

Figure 9: Synthesis of vectorised Acycloguanosine with SynB3

No cell uptake or cytotoxic effects have been measured yet.

IV – Oligonucleotides as active substances.

A 33 mer 3'-FITC-labelled synthetic oligodeoxyribonucleotide (ODN).

a) Conjugation of oligonucleotide to SynB4

A 33 mer 3'-FITC-labelled synthetic oligodeoxyribonucleotide (ODN) carrying a C6 thiol linker on its 5'end was solubilised in phosphate-buffered saline pH 8.0. The peptide SynB4 (AWSFRVSYRGISYRRSR) was activated with SPDP. The activated peptide (PySS3MP-SynB4) was solubilised in DMF. For the disulfide bridge formation, two equivalents of the peptide were incubated for 1 hour. Formation of the conjugate was assessed by mass-spectrometry. The resulting conjugate was purified by Reverse phase HPLC.

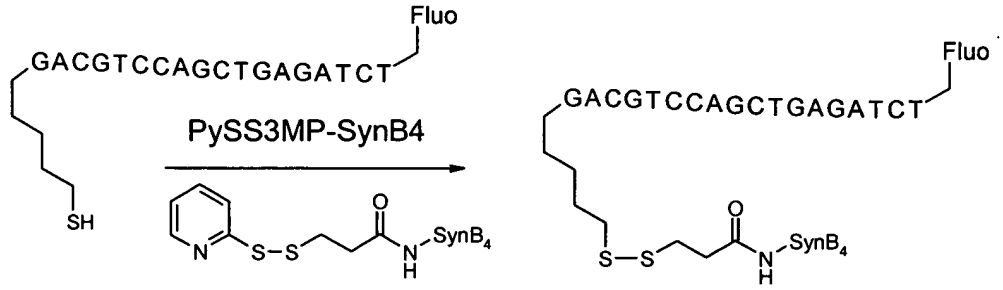

Figure 10: Synthesis of vectorised oligonucleotide with SynB4.

b) Cell uptake experiments

Cell-associated fluorescence: Free and vectorised ODN were incubated with K562 cells at 1 µM concentration and cell associated fluorescence was measured by flow cytometry.

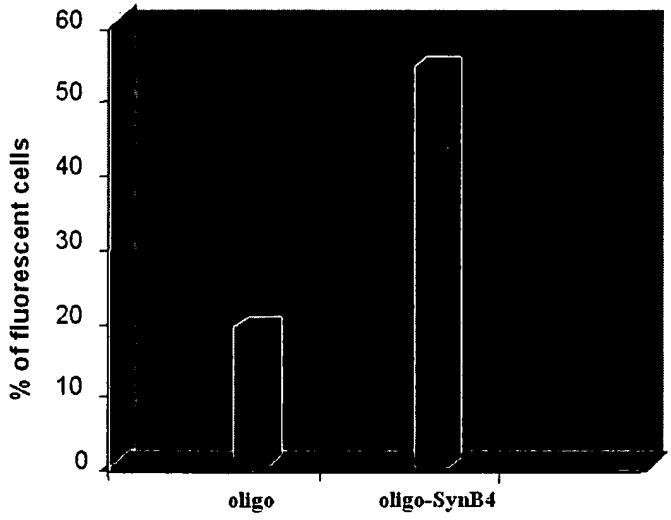

Figure 11: Cell uptake of free and vectorised oligonucleotide as measured by flow cytometry

Vectorisation of the oligonucleotide with SynB4 leads toa en enhancement of its penetration into K562 cells.

V – Chemical molecules as active substances.

A – Anti-tumorals.

1) Camptothecin.

a) Conjugation of camptothecin with SynB1

Camptothecin (CPT) was conjugated to the peptide vector SynB1 (RGGRLSYSRRRFSTSTGR) via the succinic linker using a single-pot two-step reaction. Camptothecin was first derivatized in Dimethylformamide (DMF) by succinic anhydride in the presence of Dimethylaminopyridine (DMAP) and Diisopropylethlamine (DIEA). This intermediate Camptothecin-Hemisuccinate was not isolated. The peptide vector SynB1 was coupled to Camptothecin-Hemisuccinate using PyBOP as an activator in the presence of DIEA. The resulting conjugate CPT-SUC-SynB1 was purified by Reverse-phase High Performance Liquid Chromatography.

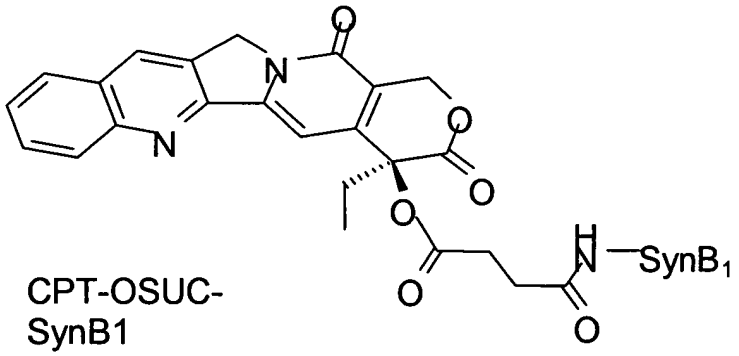

Figure 12: Structure of vectorised camptothecin with SynB1.

b) Cell uptake experiments

HT29 cells grown on microscope chamber slides were incubated for 15 min with 100 µM of either CPT-SUC-SynB1 or free Camptothecin as a control. The mean Fluorescence intensity was measured on unfixed cells using a flow cytometer using the auto-fluorecence of CPT.

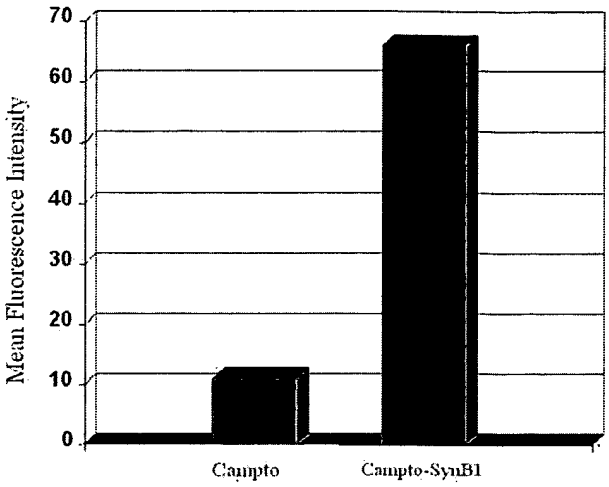

Figure 13: Cell uptake of free and vectorised camptothecin as measured by flow cytometry.

The data shows that free camptothecin has a very low uptake in HT29 cells. Vectorisation of the anticancer agent with SynB1 enhances significantly its cell uptake (6-fold).

2) Paclitaxel.

a) Conjugation of paclitaxel with SynB3

Paclitaxel (PAX) was linked to the SynB3 vector (RRLSYSRRRF) via the succinate linker in a one-pot two-steps reaction. First, the PAX hemisuccinate is formed by reaction of the primary 2' hydroxyl of PAX (1 eq) with succinic anhydride (1 eq) in the presence of catalytic amounts of diethylaminopyridine (0.1 eq), in dimethylformamide containing diisopropylethylamine (2-6 eq). The progress of this first step is monitored by reverse-phase analytic HPLC. In the second step, the peptide (1.3 eq) dissolved in DMF is added to the reaction mixture, followed by the addition of the coupling agent PyBOP (1.2 eq) dissolved in DMF. Finally, the conjugate is precipitated by addition of 10 volumes of diethylether, resuspended in H_2O/acetonitrile, purified by preparative RP-HPLC.

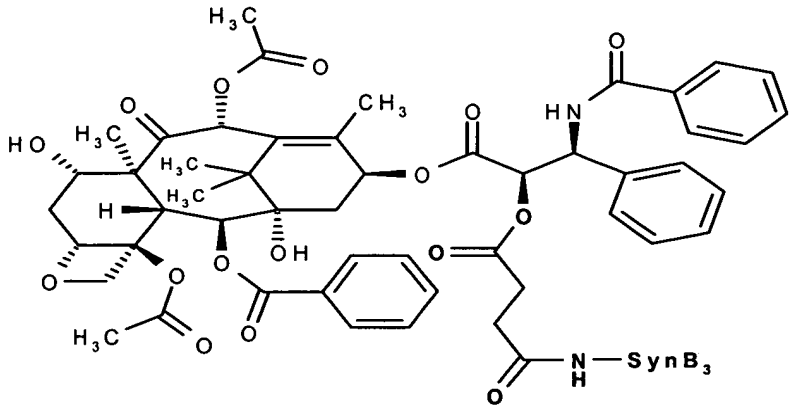

Figure 14: Structure of Pax-SynB3

b) Cell uptake experiments

Free or vectorised radioactive paclitaxel were incubated with K562/ADR cells. The cell uptake was assessed by measuring the total radioactivity associated with the cell. The data are presented as percentage of cell uptake.

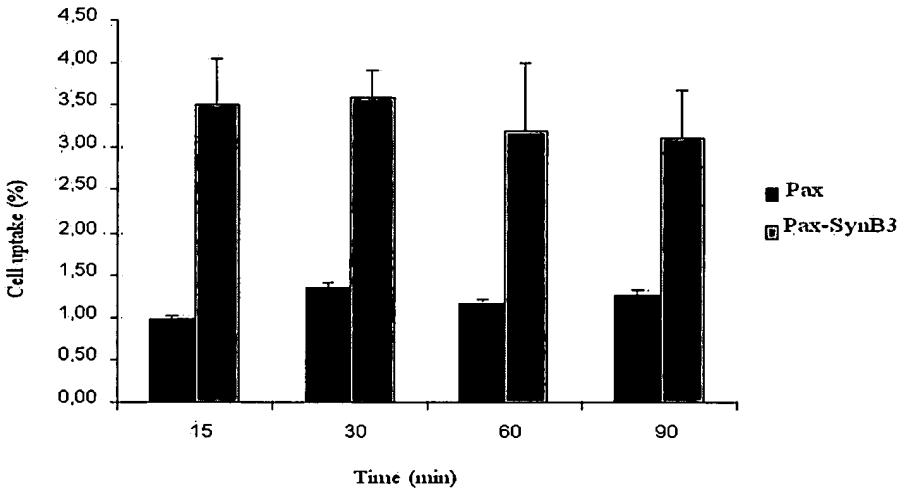

Figure 15: Cell uptake of free and vectorised radioactive paclitaxel.

The data indicates that conjugation of paclitaxel with SynB3 vector enhances significantly its uptake. At all time points, the enhancement in uptake was about 3- to 4-fold.

3) 2-Pyrrolinodoxorubicin (p-dox).

a) Construction of pDOX-OSUC-SynB3 conjugate

The free amine of doxurubicin,HCl (N9) was first protected with the Fmoc group prior to succinylation of the primary alcohol (O14), yielding N-9-fluorenylmethoxycarbonyl-14-O-succinyl-doxorubicin (FmocN-DOX-OSUC). The vector SynB3 was subsequently coupled by its free amino-terminal by activating the free carboyle of FmocN-DOX-OSUC using the pyBOP reagent, yielding FmocN-DOX-OSUC-SynB3. After debloking the DOX amine by removal of the Fmoc group with piperidine, the pyrrolino ring was formed in-situ by reaction of 4-Iodo-Butyraldehyde, yielding pDOX-OSUC-SynB3. This resulting conjugate was purified by was purified by Reverse-phase High Performance Liquid Chromatography.

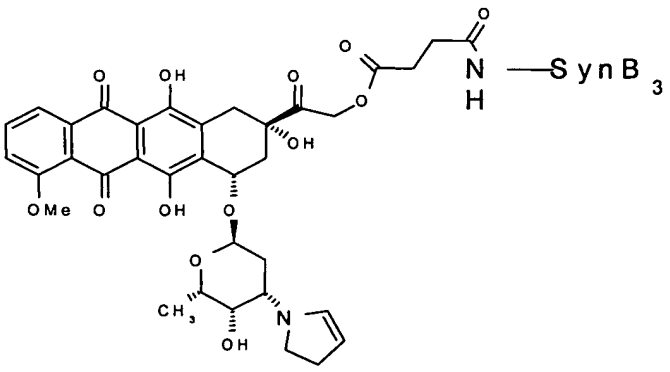

Figure 16: Structure of vectorised p-dox with SynB3

b) Cell uptake experiments

The K562 sensitive and resistant (K562/ADR) cells were incubated with increasing concentrations of the pDOX-OSUC-SynB3 conjugate. Cell uptake was measured by flow cytometry using the auto-fluorecence of p-dox.

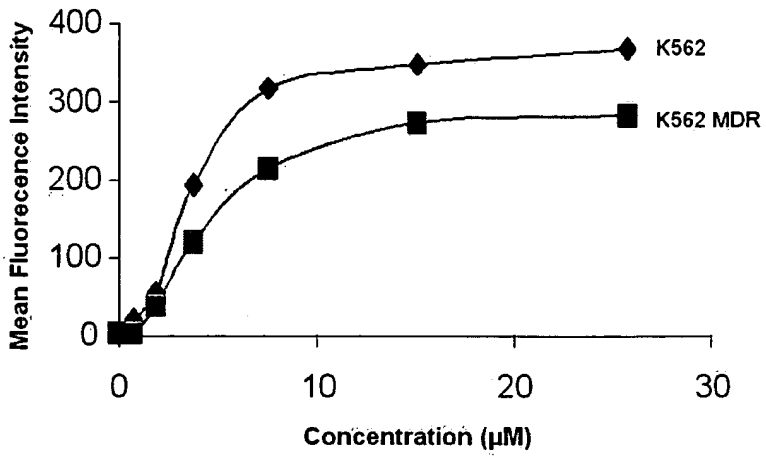

Figure 17: Cell uptake of vectorized p-dox in sensitive and resitant K562 cells.

The data presented in figure 6 indicate that vectorised p-dox penetrates in both sensitive and resistant K562 cells. This uptake was linear between 0 and 10 µM and then was saturated at higher concentrations.

B - ANTIBACTERIENS.

1) N-Benzyl-Penicillin.

a) Conjugation of N-Benzyl-penicillin to SynB1

N-Benzyl-penicillin (B-Pc, PenicillinG) was coupled to SynB1 peptide (RGGRLSYSRRRFSTSTGR) via a Glycolamidic-Ester-Linker in a two-steps one-pot reaction. The potassium salt of B-Pc (1 equivalent, compound 1) was suspended in DMF. Dibenzo-18-crown-6 (1.1 equivalent) dissolved in DMF was then added to this suspension, forming a soluble potassium chelate complex. 2,4,5-trichlorophenol bromoacetate (1 equivalent BrAcTCP), dissolved in DMF was then added to the complexed B-Pc salt, forming the ester by displacement of bromine. The reaction mixture was incubated for 1 hour at ambient temperature, and the formation of compound 4 was checked by RP-HPLC.

The peptide (1.1 equivalent) dissolved in DMF was then added to the reaction mixture, followed by ciisopropylethylamine (2 equivalents). This allowed the formation of an amide link between peptide and B-Pc. The completion of the coupling reaction was checked by RP-HPLC.

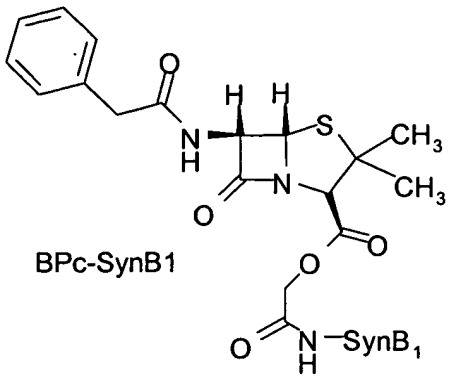

Figure 18: Schematic representation of vectorised penicillin with SynB1

b) Measure of brain uptake

The brain uptake of free or vectorised radioactive N-benzyl penicillin was measured using the in situ brain perfusion method.

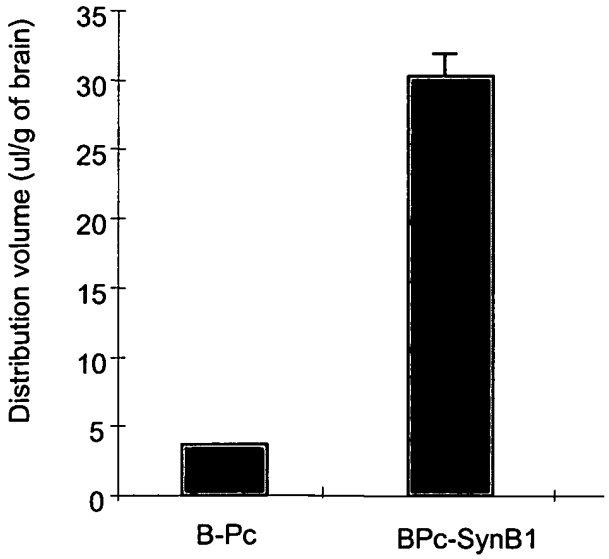

Figure 19: Brain uptake of free and vectorised N-benzyl penicillin

Figure 18 shows that vectorisation of benzyl penicillin results in a significant enhancement of its brain uptake.

2) Vancomycin.

a) Conjugation of vancomycin to SynB1

First, we chose to modify the C-terminus of the vancomycin by coupling with amine function of peptide vector SynB3 (RRLSYSRRRF) to form amide linkage (scheme). The coupling was carried out at 50°C for 30 minutes in dimethylsulfoxide solvent to increase the solubility of reactive species. The peptide bond formation requires chemical activation of the carboxyl function of the vancomycin, mediated by an activating reagent commonly employed in peptide synthesis, the benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium (PyBOP) with DIEA. This method does not require any protection of vancomycin. All of final products were obtained after purification by reverse-phase column chromatography eluted with H_2O/0.1%TFA and CH_3CN/0.1%TFA followed by lyophilisation. The chemical structures of the compounds obtained were confirmed by mass spectra and their purity was demonstrated by HPLC.

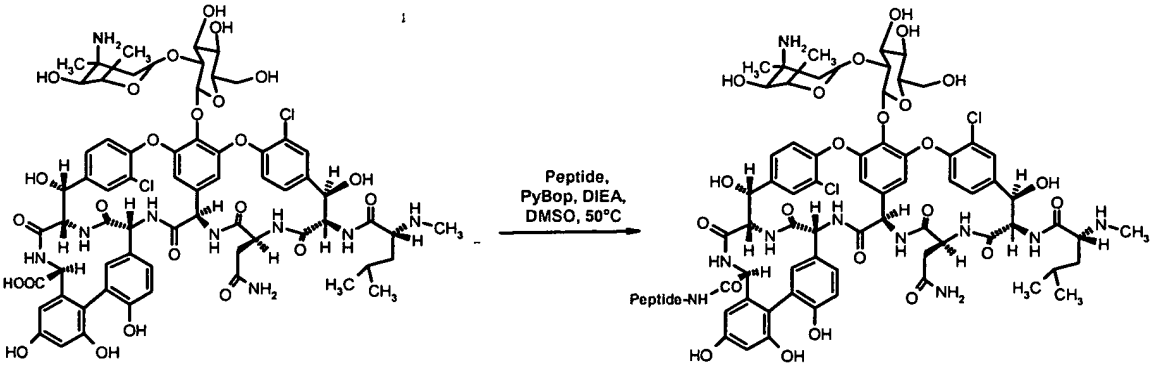

Figure 20: Scheme of the synthesis of Peptide-Vancomycin

b) Antibacterial activity:

Free and vectorised vancomycin were measured for their antibacterial effect on Methicillin-Sensitive Staphylococcus Aureus (MSSA). The cell-mediated immunity (CMI) was measured.

Compound	CMI 50 (ug/ml)
Vancomycin	1

Vancomycin-SynB3	0.17

The vectorised vancomycin was more potent than the free vancomycin indicating that the peptide vector enhanced the cell uptake of the antibiotic.